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Impairment in the aversive memory of mice in the inhibitory avoidance task but not in the elevated plus maze through intra-amygdala injections of histamine

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ABSTRACT

Rationale: Evidence indicates that histamine (HA) modulates learning and memory in different types of behavioral tasks; however, the exact nature of this modulation and its mechanisms are controversial. Furthermore, emotions are able to influence memory processing in a crucial way through the involvement of the amygdala. Our research aims to contribute to the neurobiological body of knowledge on acquisition and retrieval of emotional memory via the histaminergic amygdaloid system in mice.
Objectives: The present study investigated whether exogenous HA infused into the amygdala differentially modulates the anxiety- and fear-related memory of mice assessed by unconditioned and conditioned tasks.
Methods: Over two consecutive days, animals received bilateral microinjections of either vehicle or HA (0.1, 0.5 and 1.0 μg by 0.1 μl/side volume) into the amygdala before behavioral tests were performed. Mice were examined under two paradigms: an exposure/re-exposure procedure in the elevated plus-maze (EPM) or in the inhibitory avoidance (IA) with electric foot shock trials 1 and 2 and retention test (without foot shock).
Results: Pre-test intra-amygdala microinjection of 0.5 μg HA induced anxiolytic-like responses, but none of the three doses interfered in mnemonic processing examined in the EPM. Concerning the IA task, step-through retention latencies increased in all groups compared with their respective trials, except in the animals microinjected with 0.5 or 1.0 μg HA before the retention test. Thus, HA caused statistically significant amnesia during the session repeated 24 h after training without drugs. Retention latency was not modified by microinjections of HA both pre-trial and pre-test or by pre-trial infusion in mice subjected to IA.
Conclusions: Our data indicate that the amygdaloid histaminergic system could modulate anxiety-related behaviors in the EPM and impair the retrieval process in fear conditioning with a strong aversive stimulus. These results contribute further evidence of the distinct histaminergic influence on different emotional pathways.

1. Introduction

The histaminergic neural system (HNS) is composed of neuronal groupings in the brain of phylogenetically lower vertebrates and higher mammals that cover almost all areas of the central nervous system (CNS) (Haas and Panula, 2003). Neurons that synthesize histamine (HA) are confined to the tuberomamillary nucleus of the posterior hypothalamus (Brown et al., 2001; Watanabe et al., 1984). Moderate densities of these histaminergic fibers supply the amygdaloid complex (Haas et al., 2008; Watanabe et al., 1984), which is a set of nuclei with functional and anatomical distinction (Ehrlish et al., 2009) besides substantial HA receptor expression.

The basolateral nucleus (BLA) and central nucleus (CEA) of the amygdala are described as two functional subdivisions regarding fear conditioning (Maren, 2003; Orsini and Maren, 2012). The BLA is activated during emotional experiences that the individual perceives as anxiety, fear, stress and rage, together with cortical and subcortical afferent pathways (Rogan and LeDoux, 1996). The CEA neurons extensively project to extrinsic structures, such as the hypothalamus and the periaqueductal gray matter (Davis et al., 1994; Sah et al., 2003); instead of being a passive retransmission area from fear generating structures, the CEA is involved in the origin of aversive memory (Orsini and

Abbreviations: ABS, aversive brain system; BIS, behavioral inhibition system; BLA, basolateral nucleus of the amygdala; CEA, central nucleus of the amygdala; CNS, central nervous system; EPM, elevated plus-maze; HA, histamine; HNS, histaminergic neural system; IA, inhibitory avoidance; i.c.v., intracerebroventricular; i.p., intraperitoneally; OAE, number of open arm entries; EAE, number of enclosed arm entries; TE, total arm entries; OAT, time spent on the open arms; %OAE, percentage of entries into the open arms; %OAT, percentage of time spent on the open arms; SAL, saline; T1, first testing day; T2, second testing day.

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Maren, 2012; Zimmerman and Maren, 2010), CS-US information processing and plasticity underlying fear conditioning (Maren, 2008).

According to Panksepp (2011), one of the major emotional operating systems genetically defined and experientially refined is the fear/anxiety system. These two different states could be ethologically separated according to the defense pattern in rodents, which in turn depends on the physical distance from a threat (Blanchard et al., 1993; Davis et al., 2009). While fear is an emotion activated by imminent and real threat, anxiety is an entity aroused by distal and potential danger associated with an extended state of apprehension (Davis et al., 2009). Gross and Canteras' study (2012) supported the existence of distinct fear pathways that are specialized to address threats of an innate or learned nature, with the amygdala gathering multiple afferent inputs and distributing these inputs to their respective efferent pathways. Furthermore, the excitability of the amygdaloid circuitry can be regulated by strategies, e.g., the pharmacological approach with benzodiazepines, resulting in anxiety-reducing effects and enhancing the critical involvement of the amygdala in anxiety (Davis et al., 1994).

The physiological mechanisms underlying experience-driven changes in neural function that characterize fear conditioning could overlap with learning and memory processes (Rogan and LeDoux, 1996; McGaugh, 2004). Regarding the elevated plus-maze, a classical model to detect anxiety-like behavior, a prior maze trial can impact the emotional reaction of rodents due to the acquisition of a phobic response to the unprotected areas (Holmes and Rodgers, 1998). Known as emotional memory, these experiences with emotional valence are rapidly acquired and enable organisms to address future threats as long lasting memory (Rogan and LeDoux, 1996; Orsini and Maren, 2012). For instance, defensive responses are exhibited by animals after an aversive stimulus, which can be regulated by HA in the brain, among other neurotransmitters. Histaminergic receptor stimulation activates a pre- and post-synaptic transmitting chain implicated in neuronal plasticity and its functional and structural concomitants (Dere et al., 2010).

The acquisition and retrieval of aversive memory conditioned to taste in rats were evaluated after treatment with pyrilamine, an H1-antagonist. Microinjection of pyrilamine into the magnocellular basal nucleus impaired this aversive conditioning (Purón-Sierra et al., 2010). Recent evidence suggests that disrupted HA signaling through the H1-, H2- and H3-receptors may be an important key in cognitive disorders (Panula and Nuutinen, 2013). In pre-clinical human research, the HA level was shown to be reduced in the hippocampus, temporal cortex and hypothalamus of patients with Alzheimer's disease, which suggests that the degeneration of histaminergic neurons contributes to the cognitive decline (Airaksinen et al., 1991). Thus, HA receptors in the CNS must be studied as targets for therapeutic intervention (Passani and Blandina, 2011).

Due to the important role of the amygdala in emotional behavior and in memory modulation (Dere et al., 2010; Rogan and LeDoux, 1996), and considering that the amygdaloid histaminergic system remains unclear, the present study aims to further investigate HA function in anxiety and/or fear circuitry. We investigated the effects of exogenous HA injected into the amygdala on fear and anxiety-like behaviors, as assessed by the inhibitory avoidance task and the elevated plus-maze, respectively, as well as the emotional learning and memory of mice.

2. Material and methods

2.1. Animals

In total, 200 Swiss male mice (*Mus musculus*) aged from 5 to 7 weeks from the bioterium of the Federal University of Sao Carlos (UFSCar) were used for this experiment. Mice were kept in groups of 5 animals in polycarbonate transparent cages (28 × 18 × 11 cm) at the bioterium of the Laboratory of Neurosciences, UFSCar, until the mice reached weights between 25 and 35 g. Animals cages were stored in a ventilated rack in the same environment, with a temperature of 24 ± 1 °C, relative humidity of 55 ± 5% and under a light/dark cycle of 12/12 h (light cycle starting at 07:00 a.m.), which was automatically controlled. Water and food were available to the mice ad libitum, except during the brief testing periods. The experiments with naive mice were performed during the light phase of the photoperiod (08:00 to 14:00) to minimize the variability of the circadian influence.

The project was approved by the Ethics Committee on Animal Use (CEUA), UFSCar, under protocol number 028/2011 and registration number 018/2011. Additionally, the experimental procedures followed the rules of the National Council for Animal Experimentation Control (CONCEA), Brazil.

2.2. Behavioral tests

2.2.1. Experiment 1: effects of intra-amygdala HA microinjection on the emotional memory of mice in the inhibitory avoidance task

2.2.1.1. Inhibitory avoidance task. Studies of the HA effects on inhibitory avoidance were performed by Almeida and Izquierdo (1986) and extensively described over recent years (Benetti and Izquierdo, 2013; Bonini et al., 2011; Da Silva et al., 2006; Detrait et al., 2009). In the present investigation, IA behavior was studied in a two-trial learning, step-through situation (Fine et al., 1985; Page et al., 1991), which utilizes the natural preference of rodents for dark environments due to their photophobia (Detrait et al., 2009). A box (48 × 27 × 30 cm) composed of acrylic plates consisted of two compartments: a bright compartment (crystal colored, 456 lx luminosity, 24 × 13.5 × 15 cm) and a dark compartment (black colored, 24 × 13.5 × 15 cm), with a guillotine type door (7 × 7 cm) in the middle of the divider wall. Stainless steel grids (2.5 mm in diameter) were placed at 1-cm intervals (distance between the centers of grids) on the floor to spread out the foot shock. A camera was placed above the box and attached to a computer with software that recorded the sessions (Surveillance System GV-600; GeoVision, Inc.; Neihu District, Taipei, Taiwan).

Mice were left in the experimental room for 1 h before the beginning of the procedure, and after that, each mouse was habituated to the apparatus (Fig. 1a). Each animal was placed into the illuminated compartment facing the door, which was opened after 6 s, allowing its entry into the dark compartment. Mice that required more than 100 s to cross to the other side were eliminated from the experiment. The door was closed when the animal crossed with its four paws to the next compartment, and the mouse was taken from the dark compartment into the home cage. After 30 min, the animal was microinjected with HA or vehicle into the amygdala and singly placed in a home cage for 5 min to minimize the restraining stress. Thereafter, trial 1 was carried out by first placing the mouse in the bright compartment; after 6 s, the door was opened and a foot shock (0.5 mA, 3 s) was triggered immediately after the mouse's entry into the dark compartment. Twenty seconds later, the animal was taken out of the apparatus and returned to the home cage. A second trial (trial 2) was carried out after a 2-min interval; the criterion for successful acquisition of avoidance responses in mice was non-entry into the dark compartment for a period of 120 s. Otherwise, the mouse got a second shock if it went into the dark compartment during trial 2. Twenty-four hours after the second trial, the microinjection procedure was repeated and a retention test to evaluate avoidance response was performed. As described above, each mouse was placed in the bright side and the door was opened after 6 s; latency to re-enter the dark compartment was recorded with a maximum of 300 s. During the test, no electric shock was applied.

The apparatus was cleaned with wet cotton and 5% alcohol between the tests. The step-through latencies for entries into the dark compartment during trials 1 and 2 and the retention test were manually measured by the experimenter using a stopwatch. One to four mice per group reached up to 120 s of acquisition criteria during trial 2, i.e., mice received only one shock; other mice (on average, 80% of the total sample) received two shocks.

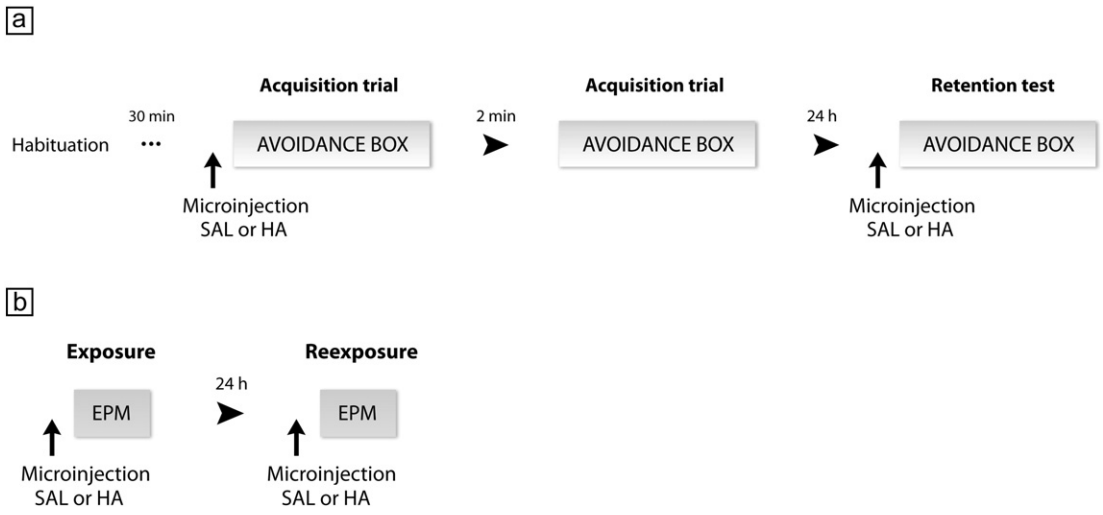

Fig. 1. Schematic representation of the experimental design for the [a] inhibitory avoidance task and [b] elevated plus maze (EPM). *HA* histamine; *SAL* saline.

2.2.2. Experiment 2: effects of intra-amygdala HA on the anxiety-like behavior and emotional memory of mice in the elevated plus-maze

2.2.2.1. Elevated plus-maze. EPM was originally proposed for rats (Pellow et al., 1985) and was later validated for Swiss mice, with some adaptations (Lister, 1987). The test is widely employed to score the anxiety level of rodents in a one-trial protocol (File, Mabbutt, Hitchcott, 1990; Carobrez and Bertoglio, 2005) and to assess emotional learning underlying the exploratory behavior in a two-trial paradigm (Rodgers et al., 1996; Dal-Cól et al., 2003; Bertoglio, Joca, Guimarães, 2006). The maze used was composed of gray acrylic plates consisting of two open arms (30 × 5.0 × 0.25 cm) and two closed arms with opaque walls (30 × 5.0 × 15.25 cm), such that the open or closed arms were opposite from each other. The arms were joined by a central platform (5 × 5 cm) and elevated 38.5 cm from the floor by a support. The perimeter of the open arms had a tiny border 0.25 cm in thickness.

A black curtain was placed around the EPM during the tests to avoid possible environmental visual clues. The luminosity at the center of the EPM was 48 lx. A camera was placed above the EPM and attached to a computer with software to record the test (Surveillance System GV-600; GeoVision, Inc.; Neihu District, Taipei, Taiwan).

During the first testing day (T1), the mice were removed from the animal house and left in the experimental room for 1 h before the test (Fig. 1b). Each mouse was microinjected with HA or vehicle and singly placed in a cage for 5 min to minimize the restraining stress. Subsequently, the animal was placed in the center of the EPM, facing one of the open arms with 5 min of free exploration. Twenty-four hours later (T2), the experimental procedure was repeated. The apparatus was cleaned with 5% alcohol between the tests.

During the test, the following conventional measurements were taken: the number of entries into the open (OAE) and enclosed (EAE) arms, the total arm entries (TE), and the time spent in the open arms (OAT). An entry was defined as all four feet in one arm. All of these parameters were measured by the experimenter using the software X-Plo-Rat (Garcia et al., 2005) for behavioral records. The number of entries and the time spent in the open arms were calculated in percentages [%OAE = (entries into the open arms/total entries) × 100; %OAT = (time spent in the open arms / 300 s) × 100]. Enclosed arm entries were considered a measurement to infer locomotor activity. In T1, increases in the percentage of open arm entries and in the percentage of time spent in the open arms were interpreted to be indicative of potential anxiolytic activity (Cruz et al., 1994; Lister, 1987; Rodgers and Johnson, 1995). In T2, a decrease in these same measurements related to T1 was interpreted to be indicative of learning and memory (Bertoglio et al., 2006; Dál-Col et al., 2003).

2.3. Surgery

Mice were intraperitoneally (i.p.) anesthetized (injection volume: 1 ml/kg) with ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight) dissolved in sterile 0.9% saline solution. Subsequently, they were positioned in a stereotaxic frame to bilaterally implant a 7-mm guide cannula (25-gauge guide) 1 mm over the amygdala according to the stereotaxic atlas for the mouse brain (Paxinos and Franklin, 2001) using the following coordinates: 0.8 mm posterior to the bregma, ±2.7 mm lateral to the sagittal midline and 3.5 mm below the skull surface. A screw was fixed anterior to the bregma, and the two cannulae were fixed with dental acrylic resin covering the mouse skull (self-polymerizing acrylic and liquid acrylic JET, Artigos Odontológicos Clássico Ltda., São Paulo, Brazil). Then, analgesic injection (flunixine meglumine, 2.5 mg/kg body weight) was intramuscularly administered and dummy cannulae (7 mm, 33 gauge) were inserted in the guide cannula to prevent blockage and contamination. Mice were allowed to recover for 3–5 days.

2.4. Microinjection and treatment

After recovery from the surgery, each mouse was microinjected with HA or vehicle (saline, SAL) 5 min before the behavioral testing. For the microinjection procedure, mice were gently restrained, the dummy cannulae were removed, and a fine needle (8 mm) was inserted into each guide cannula 1.0 mm longer than the guide end, allowing infusion of the drug into the target area. The solution was slowly injected over 60 s with the needle connected through a polyethylene tube (PE-10) to a 5.0-μl Hamilton syringe, keeping the injection unit in situ for a further 60 s. A 0.1-μl injection of HA or vehicle was controlled by a planned infusion pump (BI 2000, Insight Equipamentos Científicos Ltda., Ribeirão Preto, SP, Brazil).

Experiment 1 and Experiment 2 consisted of four groups for each dose of HA (Table 1). Both experiments were performed during 2 consecutive days with the administration of drug or vehicle prior to behavioral assessment of learning and retrieval, which are different stages of the mnemonic process. Therefore, the experimental groups were the following: control group (SAL–SAL); groups treated with HA before T1/trial 1 (HA 0.1–SAL, HA 0.5–SAL and HA 1.0–SAL) and/or before T2/retention test (HA–HA 0.1, HA–HA 0.5, HA–HA 1.0, SAL–HA

Table 1
Groups named according to histamine (HA) or vehicle (SAL) microinjection into the amygdala before T1 and T2 in the EPM and before trial 1 and the retention test in the IA. Numbers indicate the sample size for each experimental group.

	Behavioral test				Dose					
	EPM[a]		IA[b]		HA 0.1 μg		HA 0.5 μg		HA 1.0 μg	
	T1	T2	Trial 1	Test	EPM	IA	EPM	IA	EPM	IA
SAL–SAL	SAL	SAL	SAL	SAL	7	9	10	9	9	8
SAL–HA	SAL	HA	SAL	HA	9	10	9	10	7	8
HA–SAL	HA	SAL	HA	SAL	7	8	9	10	6	8
HA–HA	HA	HA	HA	HA	7	9	8	9	7	7

[a] EPM: elevated plus maze.
[b] IA: inhibitory avoidance.

0.1, SAL–HA 0.5 and SAL–HA 1.0). Animals were randomly selected on the first day of testing in both experiments.

2.5. Drugs

The powder form of histamine dihydrochloride (Sigma Chemical Co., St. Louis, Missouri, USA), 2-(4-imidazolyl) ethylamine dihydrochloride [$M_r(C_5H_9N_3 \cdot 2HCl) = 184.07$ g/mol], an HA receptor (H1–H4) agonist, were dissolved in sterile 0.9% saline solution to obtain three distinct doses of 0.1, 0.5 and 1.0 μg (Moghaddam et al., 2008; Hasenörl et al., 2001).

2.6. Histological analysis

After the behavioral assessment, all mice were subjected to euthanasia with an overdose of anesthetic solution (triple the recommended dose for xylazine and ketamine chloride dissolved in 0.9% saline solution). Brains were removed and immersed in 10% formalin for at least 3 days. Subsequently, the brains were immersed in 20% sucrose solution for 24 h and in 30% sucrose solution for a further 24 h. Thereafter, the brains were sliced into 80-μm-thick coronal sections on a cryostat-microtome (ANCAP 300). The exact microinjection sites were verified with an optic microscope (Olympus B202) and compared with the stereotactic atlas of the mouse brain (Paxinos and Franklin, 2001). Data from animals with injection sites outside the amygdala were excluded from the study.

2.7. Statistical analysis

Data are presented as the means ± standard error of the mean (SEM). After Levene's test to confirm the data homogeneity, three-way repeated measures ANOVA was applied for the mice treated with SAL or HA at its three doses in the IA and EPM. The three factors analyzed were session (for IA data: trial 1 compared with trial 2; trial 1 or trial 2 compared with test; for EPM data: T1 compared with T2), dose (comparisons among data from the doses of 0.1, 0.5 and 1.0 μg) and treatment (comparisons among the SAL–SAL, SAL–HA, HA–SAL and HA–HA groups). Duncan's post hoc test was conducted on the statistically significant F values. A probability level of less than or equal to 0.05 was accepted as significant.

All mice identically treated before trial 1 in the IA as well as in the EPM with SAL or the different doses of HA exhibited no statistically significant differences among their measurements (one-way ANOVA, $p > 0.05$). Thus, these mice were maintained in distinct groups on the first day to enable separate comparisons according to subsequent treatment 24 h later.

3. Results

Fig. 2 represents the microinjection sites that were considered successful. In this study, we did not consider specific amygdala

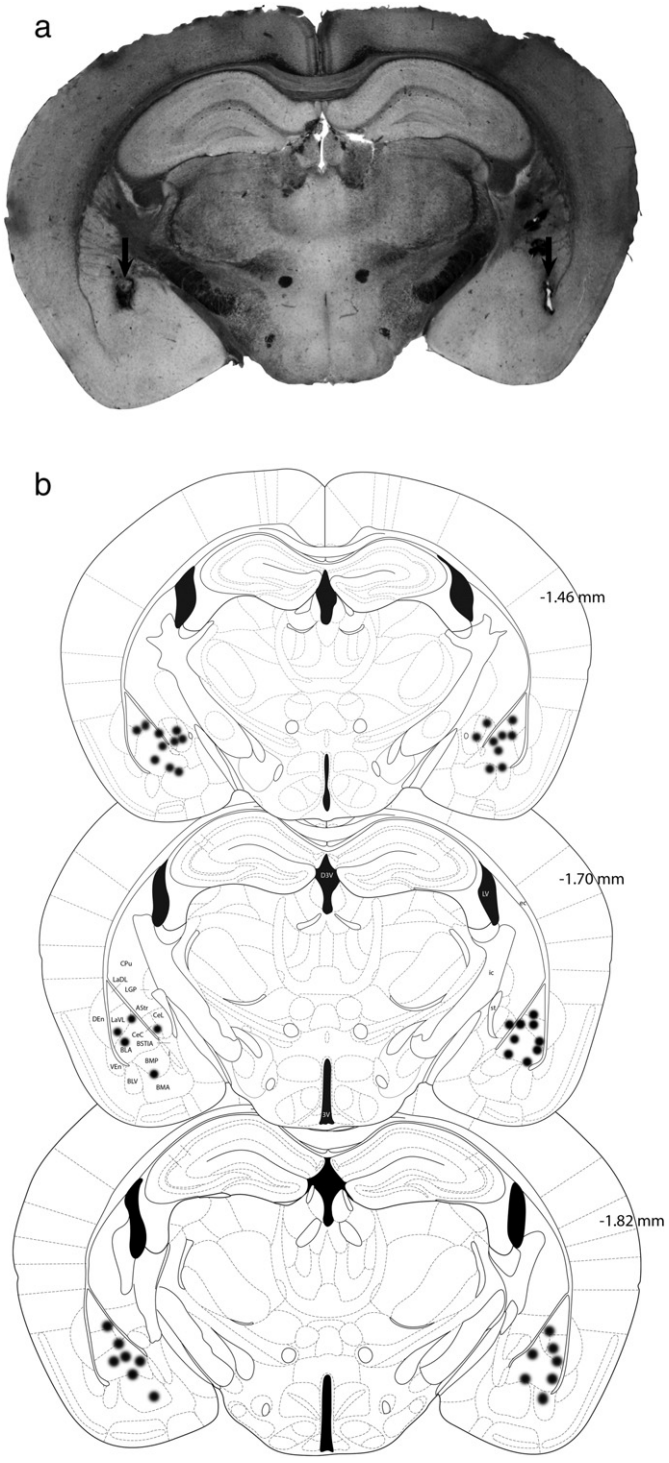

Fig. 2. Photomicrograph [a] and schematic representation of coronal sections of the mouse brain [b] showing bilateral microinjection sites into the amygdala (indicated by arrows and black dots, respectively). Diagrams are adapted from Paxinos and Franklin (2001). Rostral to caudal: 1.46, 1.70 and 1.82 mm posterior to the bregma. *AStr* amygdalostriatal transition area; *BLA* basolateral amygdaloid nucleus, anterior part; *BLV* basolateral amygdaloid nucleus, ventral part; *BMA* basomedial amygdaloid nucleus, anterior part; *BMP* basomedial amygdaloid nucleus, posterior part; *BSTIA* bed nucleus of the stria terminalis, intraamygdaloid division; *CeC* central amygdaloid nucleus, capsular part; *CeL* central amygdaloid nucleus, lateral division; *CPu* caudate putamen (striatum); *DEn* dorsal endopiriform nucleus; *D3V* dorsal 3rd ventricle; *ec* external capsule; *ic* internal capsule; *LaDL* lateral amygdaloid nucleus, dorsolateral part; *LGP* lateral globus pallidus; *LaVL* lateral amygdaloid nucleus, ventrolateral part; *LV* lateral ventricle; *st* stria terminalis; *VEn* ventral endopiriform nucleus; *3V* 3rd ventricle.

subnuclei due to the small size of the mouse amygdala and the range of the drug diffusion.

3.1. Intra-amygdala HA microinjected before testing impairs the fear memory retrieval of mice in the inhibitory avoidance task

The number of mice per group that received one or two foot shocks is described in Table 2. According to the sample, on average, 19.43 ± 4.06% of the tested mice did not enter the dark compartment during trial 2, which suggests successful learning in the first trial. However, the majority of the tested mice required two foot shocks to learn fear conditioning; all animals that received one or two shocks were tested for retention 24 h later.

Repeated measures three-way ANOVA revealed significant differences between the sessions $F(2186) = 80.76$ ($p < 0.01$) and a significant interaction between the session and treatment factors $F(6186) = 2.30$ ($p = 0.04$). Duncan's post hoc test detected that the retention test latency increased in the four groups of 0.1 μg HA related to trial 1: SAL–SAL ($p < 0.01$), SAL–HA 0.1 ($p < 0.01$), HA 0.1–SAL ($p < 0.01$) and HA–HA 0.1 ($p < 0.01$) (Fig. 3a). Furthermore, the trial 2 latency of the HA–HA 0.1 mice significantly increased compared with trial 1 latency ($p = 0.02$); this increase in the trial 2 latency was not evident for the HA 0.1–SAL group ($p = 0.11$), whose mice received the same drug and dose before trial 1.

Significant differences between sessions were also observed for the performance of mice microinjected with HA 0.5 μg. Post hoc analysis revealed that the retention test latency of SAL–SAL ($p < 0.01$), HA 0.5–SAL ($p < 0.01$) and HA–HA 0.5 ($p < 0.01$) increased when compared with trial 1 latency (Fig. 3b). Equal rising latencies were observed in the retention test compared with the respective latency in trial 2 [SAL–SAL ($p < 0.01$), HA 0.5–SAL ($p = 0.02$) and HA–HA 0.5 ($p < 0.01$)].

Duncan's test pointed to significant differences for the performed latencies of microinjected rodents with 1.0 μg HA (Fig. 3c). Latency on the retention test was greater than in trial 1 for the groups SAL–SAL ($p < 0.01$), HA 1.0–SAL ($p < 0.01$) and HA–HA 1.0 ($p < 0.01$) and greater than in trial 2 for the same groups, SAL–SAL ($p = 0.03$), HA 1.0–SAL ($p = 0.01$) and HA–HA 0.1 ($p = 0.02$). Mice in the SAL–HA groups treated with HA intra-amygdala at the 0.5 and 1.0 μg doses did not have significantly higher latencies on the retention test than trials 1 and 2 ($p > 0.05$).

In addition to the absence of a difference with treatment $F(3,93) = 2.16$ ($p = 0.1$) as a single variable, a difference for the interaction between session and treatment is indicated by the three-way ANOVA $F(6186) = 2.30$ ($p = 0.04$). Duncan's test identified higher latencies during the retention test in the control ($p < 0.01$), HA 0.5–SAL ($p = 0.01$) and HA–HA 0.5 ($p < 0.01$) groups than in the SAL–HA 0.5 (Fig. 3b). Furthermore, on retention latencies, this measure was also significantly increased in the control ($p = 0.04$), HA 1.0–SAL ($p < 0.01$) and HA–HA 1.0 ($p = 0.03$) groups than in the SAL–HA 1.0 (Fig. 3c). These differences among treatment groups during the test are not shown in the graphic representation. These results suggest that the experimental groups treated with vehicle pre-trial 1 and HA at the doses of 0.5 or 1.0 μg pre-test did not evoke aversive memory.

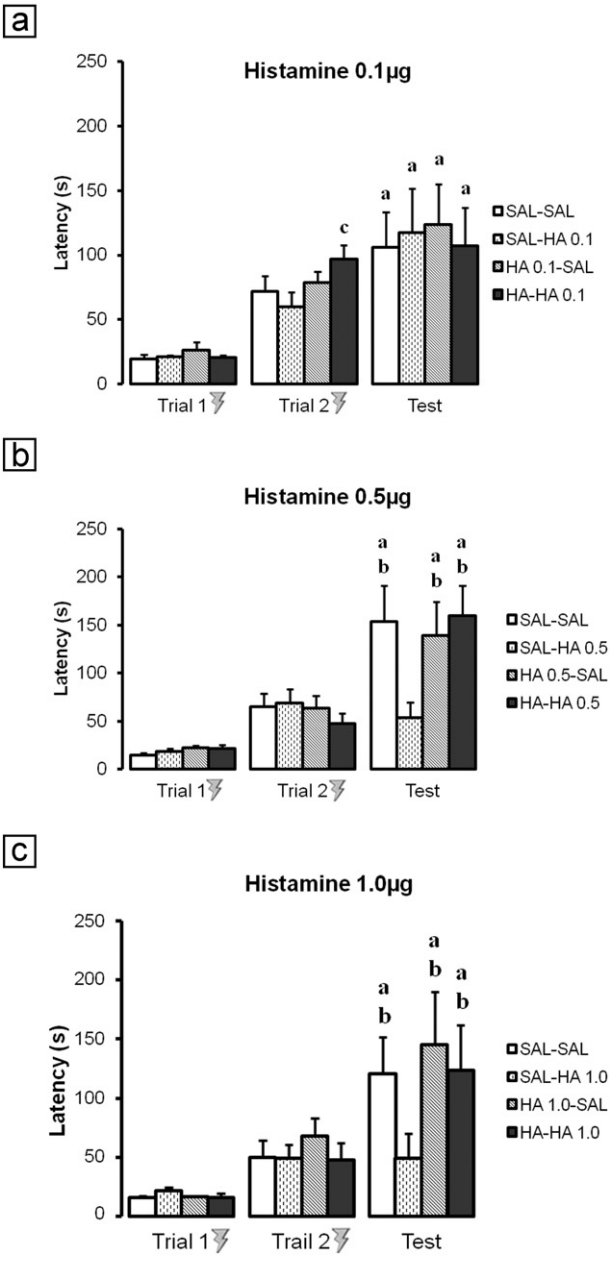

Fig. 3. Amnesic effect of histamine — tested with the doses 0.1, 0.5 and 1.0 μg/0.1 μl per side — bilaterally microinjected into the mouse amygdala on inhibitory avoidance memory. Columns represent means ± SEM of the latencies obtained from analyzed mice: [a] SAL–SAL (n = 9), SAL–HA 0.1 (n = 10), HA 0.1–SAL (n = 8) and HA–HA0.1 (n = 9); [b] SAL–SAL (n = 9), SAL–HA 0.5 (n = 10), HA 0.5–SAL (n = 10) and HA–HA 0.5 (n = 9); [c] SAL–SAL (n = 8), SAL–HA 1.0 (n = 8), HA 1.0–SAL (n = 8) and HA–HA 1.0 (n = 7). [a]$p \leq 0.05$ retention test compared with trial 1, [b]$p \leq 0.05$ retention test compared with trial 2, [c]$p \leq 0.05$ trial 2 compared with trial 1. *HA* histamine; *SAL* saline.

Table 2
Number of mice per group that received one or two foot shocks during the two-trial learning of the inhibitory avoidance task.

	Number of mice that received					
	1 ft shock			2 ft shocks		
Inhibitory avoidance group	0.1 μg	0.5 μg	1.0 μg	0.1 μg	0.5 μg	1.0 μg
SAL–SAL	2	2	1	7	7	7
SAL–HA	1	4	0	9	6	8
HA–SAL	1	3	2	7	7	6
HA–HA	4	0	1	5	9	6

HA: histamine; SAL: saline.

3.2. Intra-amygdala HA microinjected before testing does not affect the anxiety-like behavior and emotional memory of mice in the elevated plus-maze

Enclosed arm entries (EAE) are considered a locomotor activity measurement in the EPM (Fig. 4a, d, g). In addition to the absence of an interaction difference, the three-way ANOVA revealed a significant difference for dose $F(2,83) = 5.46$ $p < 0.01$ in EAE measurement during T1. Duncan's test identified a significant decrease in the EAE frequency during T1 in the HA 1.0–SAL group compared with control ($p = 0.02$) and SAL–HA 0.5 ($p = 0.05$) — both groups from the intermediate

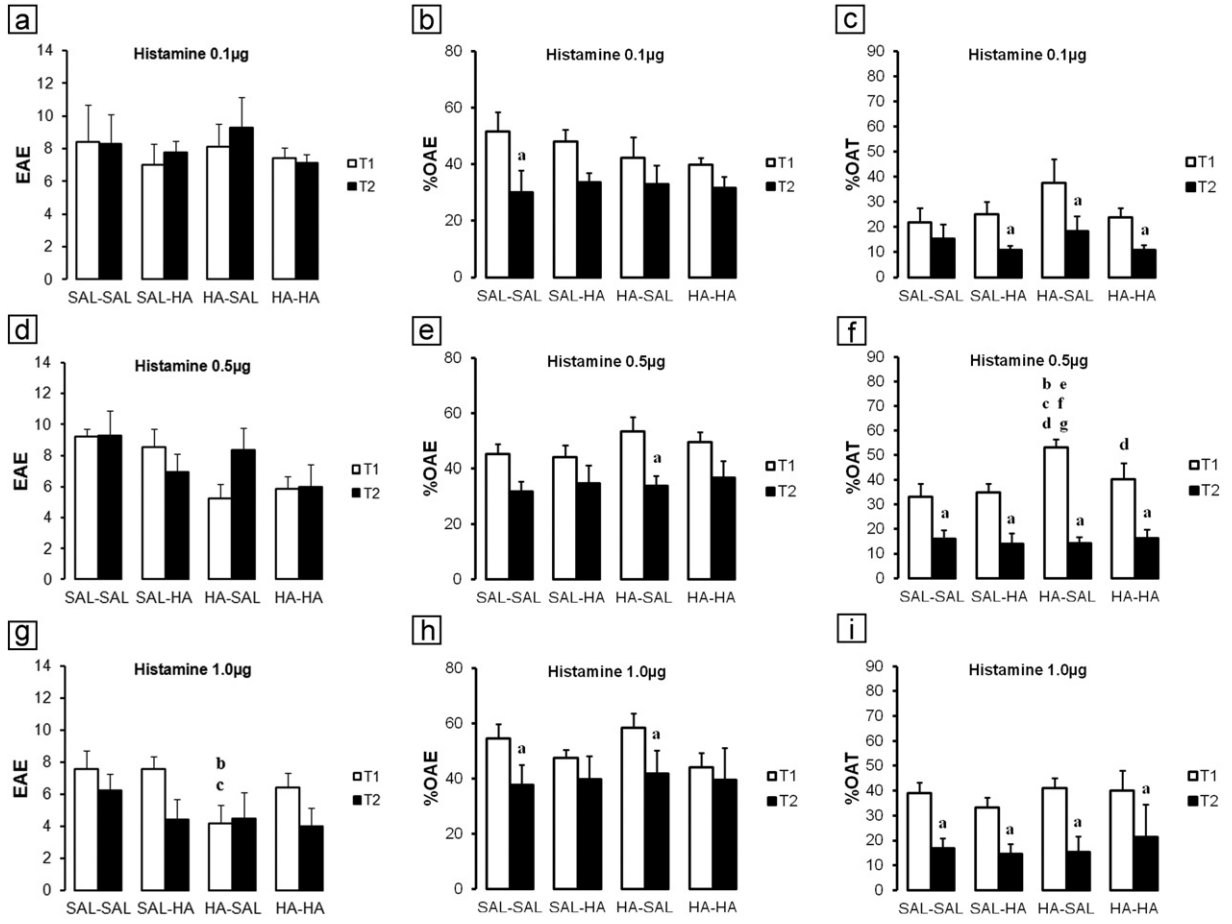

Fig. 4. Anxiolytic-like effect of histamine (0.5 μg/0.1 μl per side) bilaterally microinjected into the mouse amygdala, but its three doses (0.1, 0.5 and 1.0 μg/0.1 μl per side) do not affect emotional memory. Columns represent means ± SEM from (a, d and g) frequency of enclosed arm entries (EAE), (b, e and h) the percentage of open arm entries (%OAE) and (c, f and i) the percentage of time spent in open arms (%OAT) [SAL–SAL (n = 7), SAL–HA 0.1 (n = 9), HA 0.1–SAL (n = 7) and HA–HA 0.1 (n = 7); SAL–SAL (n = 10), SAL–HA 0.5 (n = 9), HA 0.5–SAL (n = 9) and HA–HA 0.5 (n = 8); SAL–SAL (n = 9), SAL–HA 1.0 (n = 7), HA 1.0–SAL (n = 6) and HA–HA 1.0 (n = 7)]. $p \leq 0.05$ [a]T2 compared with T1; [b]compared with SAL–SAL 0.5 in T1; [c]compared with SAL–HA 0.5 in T1; [d]compared with SAL–SAL 0.1 in T1; [e]compared with SAL–HA 0.1 in T1; [f]compared with HA–HA 0.1 in T1; [g]compared with SAL–HA 1.0 in T1. *HA* histamine; *SAL* saline; *T1* trial 1; *T2* trial 2.

dose. Furthermore, in T2, decreased EAE was observed in SAL–HA 1.0 ($p = 0.03$), HA 1.0–SAL ($p = 0.03$) and HA–HA 1.0 ($p = 0.02$) of the highest dose compared with HA 0.1–SAL mice of the lower dose and compared with SAL–SAL of the intermediate dose.

Total entries (TE) were also analyzed tentatively to clarify the general locomotion results (Table 3). According to ANOVA, there was an interaction between session and treatment $F(3,83) = 2.83$ ($p = 0.04$) and a significant difference between T1 and T2 $F(1,83) = 17.42$ ($p < 0.01$). This suggests that the TE data from the first and second testing days are influenced by HA injections. Post hoc analysis reveals that the SAL–HA 1.0 ($p < 0.01$) and HA–HA 1.0 ($p = 0.03$) mice present lower TE during re-exposure than in T1. Taken together, the dose difference for EAE in T1 and the interaction between session and treatment for TE of 1.0 μg HA suggest a tendency of locomotor hypoactivity in mice treated with the highest dose of HA; not all animals identically treated with the highest dose exhibited these differences.

Regarding the measurements of anxiety-like responses, three-way ANOVA did not reveal a significant difference for the %OAE comparisons for dose, treatment or interactions in the microinjected groups with HA 0.1 μg, 0.5 μg and 1.0 μg ($p > 0.05$). ANOVA applied to the %OAE of tested groups indicated a significant difference between T1 and T2 $F(1,83) = 42.68$ ($p < 0.01$). This difference for %OAE between exposure and re-exposure was described by Duncan's post hoc test in mice treated with SAL–SAL (controls of the lowest, $p = 0.01$, and highest doses,

Table 3
Conventional measurements of the mice with histamine (HA 0.1, 0.5 and 1.0 μg/0.1 μl per side) or vehicle (SAL) microinjected into the amygdala and analyzed in the EPM. Values represent mean ± SEM from frequency of enclosed arm entries (EAE) and total entries (TE) during T1 and T2.

Variable	Control		Treatment					
	SAL/T1	SAL/T2	SAL/T1	HA 0.1/T2	HA 0.1/T1	SAL/T2	HA 0.1/T1	HA 0.1/T2
Total entries (TE)	15.57 ± 3.32	13.14 ± 3.26	12.67 ± 1.99	11.67 ± 0.85	15.00 ± 2.62	15.57 ± 2.84	12.43 ± 1.09	10.86 ± 1.16
	SAL/T1	SAL/T2	SAL/T1	HA 0.5/T2	HA 0.5/T1	SAL/T2	HA 0.5/T1	HA 0.5/T2
	17.20 ± 1.12	14.00 ± 2.46	15.56 ± 1.66	10.56 ± 1.84	11.89 ± 2.19	12.89 ± 2.03	11.38 ± 0.96	10.25 ± 2.44
	SAL/T1	SAL/T2	SAL/T1	HA 1.0/T2	HA 1.0/T1	SAL/T2	HA 1.0/T1	HA 1.0/T2
	16.11 ± 1.20	11.33 ± 2.03	14.43 ± 1.32	7.29 ± 1.66[a]	9.33 ± 1.93	9.00 ± 3.51	11.71 ± 2.17	6.29 ± 1.54[a]

[a] $p \leq 0.05$ compared with T1.

$p = 0.04$, respectively), HA 0.5–SAL ($p = 0.02$) and HA 1.0–SAL ($p = 0.04$). Fig. 4b, e and h presents the significant decreases in %OAE during the re-exposure compared with the exposure values.

According to ANOVA, there is a marked interaction between session and treatment factors $F(3,83) = 3.73$ ($p = 0.01$) for the %OAT during T1, although no significant difference for treatment was revealed. Duncan's test (Fig. 4e) points to the elevated %OAT during T1 for the HA 0.5–SAL group compared with the control ($p = 0.02$) and SAL–HA 0.5 ($p = 0.04$) groups.

Three-way ANOVA also detected an interaction between session and dose $F(2,83) = 6.29$ ($p < 0.01$) on the %OAT. Significant differences regarding dose $F(2,83) = 3.02$ ($p = 0.05$) and testing days $F(1,83) = 196.95$ ($p < 0.01$) were revealed by ANOVA. For the exposure analysis, post hoc test indicated that HA 0.5–SAL ($p < 0.01$) and HA–HA 0.5 ($p = 0.04$) exhibited more time spent in the open arms than the control group from the HA 0.1-μg experiment. Furthermore, HA 0.5–SAL mice had an increased %OAT in T1 compared with the SAL–HA 0.1 ($p < 0.01$), HA–HA 0.1 ($p < 0.01$) and SAL–HA 1.0 ($p = 0.02$). Thus, there was a tendency toward an anxiolytic action of HA at the dose of 0.5 μg because the drug altered %OAT without changes in %OAE measurement.

Regarding the %OAT measurements, decreased re-exposure responses in T2 related to T1 were identified for the following groups by Duncan's post hoc test: mice treated with HA 0.1 μg [SAL–HA 0.1 ($p = 0.02$), HA 0.1–SAL ($p < 0.01$) and HA–HA 0.1 ($p = 0.03$)] (Fig. 4c); mice treated with HA 0.5 μg [control ($p < 0.01$), SAL–HA 0.5 ($p < 0.01$), HA 0.5–SAL ($p < 0.01$) and HA–HA 0.5 ($p < 0.01$)] (Fig. 4f); and mice treated with HA 1.0 μg [control ($p < 0.01$), SAL–HA 1.0 ($p < 0.01$), HA 1.0–SAL ($p < 0.01$) and HA–HA 1.0 ($p < 0.01$)] (Fig. 4i). Considering the reductions in %OAE of some groups and %OAT in most groups during the retest, HA microinjection into the mouse amygdala was not able to modify the mnemonic processes examined in the EPM.

4. Discussion

The inspection of brain sections confirmed the microinjection sites in the amygdala. We could not establish a correspondence between behavioral responses and the excitation of specific sub-nuclei or antero-posterior divisions due to the great proximity of the neuronal groups on a sub-nuclei level. However, it is well known that CEA is a neural nucleus that mediates amygdaloid outputs (Orsini and Maren, 2012), strongly related to contextual fear and memory formation (Zimmerman and Maren, 2010), besides the associative function of the external stimulus of threatening or learning under stressful conditions mediated by BLA neuronal networks (McGaugh, 2004; Orsini and Maren, 2012). BLA and CEA were the main sites of the mouse amygdala where exogenous HA was infused in this study.

The time interval (5 to 14 min) between the microinjection and the test in the IA and in the EPM was appropriated to stimulate amygdaloid activity. Exogenous HA could influence the behavioral responses exhibited by the mice during the experiments because the apparent half-life of this modulatory neurotransmitter in the brain is approximately 20 min (Schwartz et al., 1986).

4.1. Intra-amygdala HA microinjected before testing impairs the fear memory retrieval of mice in the inhibitory avoidance task

In the IA task, the animal learns to associate the apparatus context, which is not initially aversive, to an electric foot shock (Cahill and McGaugh, 1998). During the trial, rodents experience the pairing of a prior neutral stimulus, the posterior conditioned stimulus (CS), which is composed of the dark compartment context and its entry, with an unconditioned stimulus (US), the foot shock (Blake et al., 2008). In a subsequent trial, these animals avoid the black compartment due to the inhibition of innate mouse behavior, which is to look for dark burrows. Fear elicited by exposure to a cue or context associated with foot shock is a phenomenon called fear conditioning (Gross and Canteras, 2012). Robust avoidance responses exist because of fear and have critical functions to individual survival, such as protection against a potentially threatening environment (Fendt and Fenselow, 1999).

HA microinjections at 0.5 and 1.0 μg into the amygdala before the retention test impair memory retrieval, as observed in decreased latencies for SAL–HA 0.5 and SAL–HA 1.0 mice. The amnesic effect is indicated by the comparison among the lower test latencies of those groups, in relation to its respective trial latency, and the higher test latencies of control and HA–HA mice. These latter groups exhibit fear conditioning, which is inferred from increased retention latencies. Thus, intra-amygdala administration of HA blocks the evocation of aversive information acquired with endogenous histaminergic levels. The present result is supported by the drug's influence on sessions according to ANOVA interaction. It is unlikely that HA impaired the acquisition because there was no difference between the saline- and HA-treated groups in trials 1 and 2.

Former studies have also demonstrated the inhibitory role of HA on the consolidation phase through H1 or H2 subtypes. Rats microinjected i.c.v. with HA after a one-trial step-through exhibited an impairment of memory retention; this deficit was attenuated by pyrilamine (H1-blocker) and cimetidine (H2-blocker) (Zarrindast et al., 2002). On H1 receptors, Hasenhörl et al. (2001) suggested a retention improvement by chlorpheniramine infusions into the nucleus accumbens in rats examined on the uphill avoidance test. Hence, blocking histaminergic neurotransmission via H1 receptors could result in facilitation of the mnemonic process.

Neuromodulatory interactions in the amygdala, as experimentally reported and discussed by McGaugh (2004), have a crucial role in emotional memory processing, not only in the BLA but also in the CEA (Orsini and Maren, 2012; Zimmerman and Maren, 2010). We suggest that the HA modulation of the intrinsic cholinergic and/or glutamatergic neurotransmission in BLA and CEA nuclei may justify the amnesic effect observed. Exogenous HA would increase its availability in the amygdaloid network; the action of H1 and H2 receptors would inhibit acetylcholine (ACh) release in such a way as to disturb the local neuroplasticity and to cause a memory deficit for the mice tested. Passani et al. (2001) treated rats with different H3-antagonists intra-BLA in the post-trial of contextual fear and verified a reduction in ACh release that compromised memory retention. Additionally, the highest doses of HA could activate the H3 auto- and heteroreceptor in glutamatergic neurons, which compose most of the amygdala (approximately 80%) and underlie synaptic plasticity (Sah et al., 2003), promoting a negative feedback and reducing the action of glutamatergic receptors on long-term potentiation (LTP).

In the present study, HA at 0.5 or 1.0 μg increases the neurotransmitter concentration at the amygdala, which affects the excitatory synapses during aversive event retrieval. These physiologically disturbed concentrations could correspond to a pathological HA release, which destabilizes the transmission and impairs the mice's ability to remember the shock experience. Therefore, HA and its receptor(s) activation may be tonically involved in very specific aspects of fear and anxiety behaviors during aversive re-exposure. A high HA level may mimic the effects of anxiolytic stimuli in the EPM and impair the IA retrieval.

4.2. Intra-amygdala HA microinjected before testing induces an anxiolytic-like effect but does not affect the emotional memory of mice in the elevated plus-maze

The EPM has been traditionally used to investigate anxiety-like behaviors and, most recently, emotional memory. Evidence has confirmed that a rodent's previous experience in this apparatus modifies subsequent reactions to the same test (Holmes and Rodgers, 1998; Rodgers et al., 1996). Thus, there is a qualitative modification of the emotional status that changes from an unconditioned (T1) to a learned form

(T2) of emotional responses (Bertoglio and Carobrez, 2000), with anxiety evaluation in T1 and fear behaviors observed in T2. Mice that are experienced with the EPM will exhibit an elevated avoidance of the potentially dangerous arms of the maze based on their thigmotaxis and vision.

The dose of 0.5 μg HA into the amygdala elevates the conventional measure of %OAT during T1, which could be clearly observed in the HA 0.5–SAL mice and, to a lesser extent, in HA–HA 0.5, suggesting an anxiolytic-like effect on murine exploration. This anxiolysis does not occur due to any motor alterations because the treatment does not alter the number of entries into the enclosed arms of the EPM or the total frequency of entries during T1. However, the EAE measurement in T1 is reduced in mice treated with the highest dose (HA 1.0–SAL group), which indicates a tendency of locomotor activity impairment, even though it does not occur for HA–HA 1.0 animals.

Studies have already shown a histaminergic modulation of anxiety examined in the EPM and in other models with pharmacological as well as genotype manipulations. HA administration into the hippocampal CA1 of rats, a limbic area that underlies the defensive response, provoked an anxiolytic effect assessed in the EPM (Zarrindast et al., 2006). Knockout of the H3 receptor produced reduced anxiety-like behaviors related to the wild-type controls in the EPM and in the elevated zero maze, likely due to an increased release of HA and subsequent stimulation of the other histaminergic receptors (Risk et al., 2004).

In contrast, intra-amygdala administration of HA induced anxiogenic-like effects in rats examined in the EPM exposure, while the H1-antagonist pyrilamine administration provoked anxiolytic effects (Moghaddam et al., 2008) and reversion of the anxiogenesis (Zarrindast et al., 2005). Benetti and Izquierdo (2013) suggested that intra-amygdala administration of HA, agonists and antagonists for distinct histaminergic receptors in rats 24 h before the test did not influence the performance during the exposure in the EPM. Those findings suggest that the role of HNS on anxiety-like responses depends on the action of a specific receptor and subject.

According to the test/retest paradigm, the present results suggest a decreased exploration of the unprotected areas in T2, as well as the natural condition; that is, there are no effects of intra-amygdala HA on the emotional memory of mice subjected to the EPM. The OAE of the control and the groups treated with HA 0.5 or 1.0 μg pre-T2 and treated with HA 1.0 before the two tests decreased significantly during the re-exposure, compared with the respective exposure. A similar reduction was observed for %OAE in T2 of the groups at the lowest dose. However, the number of entries was less indicative of avoidance than the time in the open arms; we considered the significant reductions in OAT and %OAT and important increases in CAT in T2 as decisive measures to establish successful learning and memorization of the aversive context. Therefore, in the present study, the effects of HA microinjection into the mouse amygdala are absent in the T1/T2 paradigm.

4.3. Comparison between Experiment 1 and Experiment 2

The two experimental models used in this study evaluated the aversive memory of mice but in the following distinct behavioral paradigms: the EPM is based on the innate avoidance of potentially threatening areas acquired on the first test, and the IA is based on the conditioned avoidance to a real danger and is related to fear expression. Aversiveness memory is modulated by more than one neuroanatomical system with different HA amounts operating on each one of these pathways, as reported by Fiorenza and colleagues (2012) for the extinction stage in fear-motivated tasks, contextual conditioned fear and IA.

Distinct neural systems control the categories of emotion, e.g., anxiety and fear. The circuit associated with the unconditioned defensive response is known as the aversive brain system (ABS) (Brandão et al., 1999, 2003), whereas the learned behavior is arranged by the behavioral inhibition system (BIS) (Gray and McNaughton, 2000). Similar to an interface between sensations and emotions, the amygdaloid complex seems to be involved in both systems (LeDoux, 2000), processing the information through an intrinsic network (intra and internuclear projections) according to the interactions with other integrative areas of sensitive inputs (Sah et al., 2003). The BLA would be the local host for critical interactions among neuromodulator systems that regulate extrinsic mnemonic processes of various experiences (Davis et al., 2003); one of these modulator systems could be the HNS (Jiang et al., 2005; McGaugh, 2004).

McNaughton and Corr (2004) proposed the existence of functional, behavioral and pharmacological distinctions between anxiety and fear. Fear would move the animal away from a dangerous source; fear induces fight, flight and freezing behaviors. In contrast, anxiety occurs in an approach-avoidance conflict to move the animal toward the threat source; anxiety induces the inhibition of approach and avoidance, increases the risk assessment and produces defensive quiescence. The equivalent and concurrent activation of the ABS and approach systems creates an anxiety status, along with an importantly parallel participation of the BIS in the expression of risk access postures and elevated aversion during conflict situations (Gray and McNaughton, 2000; McNaughton and Corr, 2004). The HNS could promote an important mechanism to refine an appropriated behavioral response processed by the amygdala (Benetti and Izquierdo, 2013).

Our research group previously showed that the cerebellar histaminergic system is involved in the consolidation stage differently when the emotional memory has an anxiety component (EPM) (Gianlorenço, Canto-de-Souza and Mattioli, 2011) or a fear component (IA) (Gianlorenço, Canto-de-Souza and Mattioli, 2013). Considering that two different emotional systems — the ABS and BIS — are involved in anxiety and fear learning, we suggest that the histaminergic neurons in the amygdala modulate these emotional circuitries in different ways. HA is involved in memory disruption, which is linked to aging, e.g., Alzheimer's disease (Alvarez et al., 1996; Passani and Blandina, 2011). Thus, these present findings contribute to the biological basis of investigation for learning and memory and, consequently, to a better comprehension of emotional and mnemonic disorders.

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Contributors

Fernanda Daher conducted the literature search, data collection and wrote the first draft of the manuscript. Prof Rosana Mattioli reviewed the manuscript, providing feedback and corrections. All of the authors contributed to the original idea of this research, analysis and interpretation of the data, and approved the final manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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